Restriction of p416-TEF1 as preparation for Gibson assembly

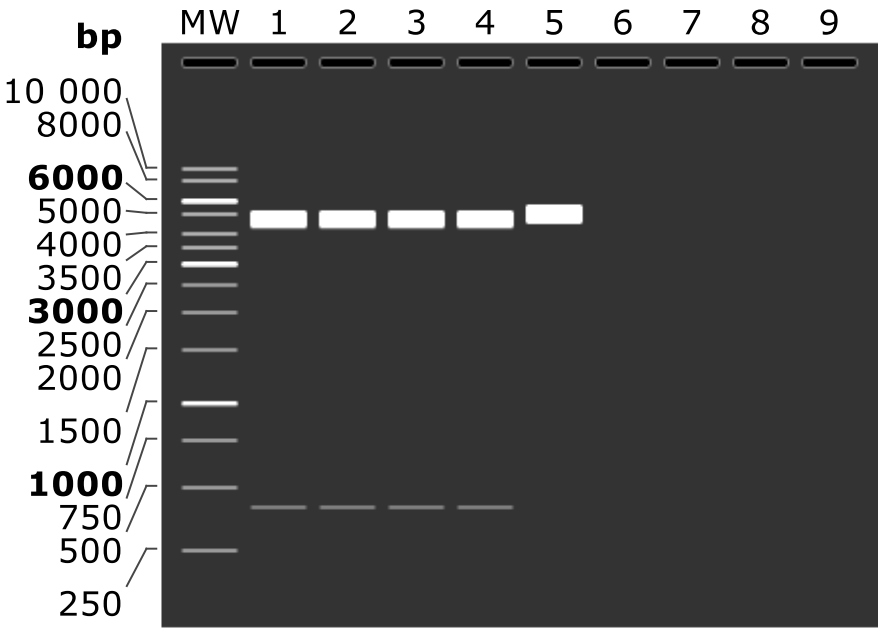
Cut minipreped p416tef with XbaI and SacI

	Plasmid
Sterile MQ water	Up to 20 µL
10X FastDigest Green Buffer	2 µL
p416tef1	Up to 1000 ng
XbaI	1 µL
SacI	1 µL
Total	20 µL

Control: cut p416tef with XbaI

	Plasmid
Sterile MQ water	Up to 20 µL
10X FastDigest Green Buffer	2 µL
p416tef	Up to 500 ng
XbaI	1 µL
Total	20 µL

- Incubate for 30 minutes at 37 degrees (SacI needs 15 min, no unspecific cutting of both enzymes below 16h incubation)
- Cast gel without GelGreen
- Load 5 µL of double digested product (in lane 1-4, like below).
- Load 10 µL of single digested product (in lane 5, like below).
- Run on gel 80V 1h10min
- Post stain with GelGreen in MQ water for 20 minutes.
  Expected sizes for restriction: 5113 and 415 bp.
  Expected size for control: 5528 bp.
  Expected sizes are visualized below:



- Pre-weigh an empty eppendorph tube to be used for gel extraction.
  Cut out 5113 bp band from lane 1-4 and purify using Gene Jet™ gel extraction kit.
- Elute with 30 µL elution buffer instead of 50 µL to get higher concentration
- Measure concentration and purity of cut plasmid using nanodrop™.

pAQR1

Prepare Gibson reaction

1. **p416+pAQR1+GFP**

	p416tef1 cut	pAQR1	GFP	Mastermix	Sterile MQ water
Conc fragment (ng/µL)	24.2	27,2	35	---	---
Volume (µL)	5	3	2	10	0

Gibson reaction
2 hours 50 degrees

Transformation to *E. coli* (total of 5 tubes of E. coli)
Refer to e. coli heat shock transformation protocol.
Use SOC instead of LB for the recovery stage after heat chock.

- Transforms 5 µL of Gibson assembly to *E. coli*: construct and positive control.
- Also transform 2 µL of Gibson assembly: construct and positive control
- Do a negative control: transform with 5 µL of 10X diluted double digested p416tef1 (10X dilution: 1 µL double digested p416tef1 + 9 µL sterile MQ water). This will transform 25 ng of linear empty plasmid.
- Plate on total five LB + amp plates (2x construct + 2x positive control + 1x negative control)

Replate and inoculate colony overnight

Replate 8 colonies (depending on amount on negative control) on 1 LB + amp plate. Dip green loop in liquid LB + amp media after you replated the colony. Number plates and tubes. Inoculate liquid tubes overnight 37 degrees.

Miniprep
Check which plates has colonies and take corresponding tubes for miniprep. Follow protocol. Spin longer with the column empty to remove ethanol. Elute with 50 µL elution buffer.

Restriction verify
See last page

pFBP1

Prepare Gibson reaction

p416+pFBP1+GFP

	p416tef1 cut	pFBP1	GFP	Mastermix	Sterile MQ water
Conc fragment (ng/µL)	24.2	14,1	35	---	---
Volume (µL)	4	4,5	1,5	10	0

Gibson reaction
2 hours 50 degrees

Transform to *E. coli* (total of 3 tubes of E. coli)
Refer to e. coli heat shock transformation protocol.

- Transforms 2 µL of Gibson assembly to *E. coli.* Do duplicate! Add 150 µL SOC to one and 250 µL to the other.
- Negative control: 2 µL of 5X diluted plasmid (5X dilution: 1 µL double digested p416tef1 + 4 µL sterile MQ water). This will transform 9,68 ng of linear empty plasmid.
- Plate both on LB + amp
- 37 degrees overnight

Replate and inoculate colony overnight

Replate 4 colonies (depending on amount on negative control) on 1 LB + amp plate. Dip green loop in liquid LB + amp media after you replated the colony. Number plates and tubes. Inoculate liquid tube overnight 37 degrees.

Miniprep
Check which plates has colonies and take corresponding tubes for miniprep. Follow miniprep protocol. Spin longer with the column empty to remove ethanol. Elute with 50 µL elution buffer.

Restriction verify
See last page

pGLN1

Prepare Gibson reaction

p416+pGLN1+GFP

	p416tef1 cut	pGLN1	GFP	Mastermix	Sterile MQ water
Conc fragment (ng/µL)	24.2	82,6	35	---	---
Volume (µL)	4	1	2	10	3

Gibson reaction
2 hours 50 degrees

Transform to *E. coli* (3 tubes of E. coli)
Refer to e. coli heat shock transformation protocol.

- Transforms 2 µL of Gibson assembly to *E. coli.* Do duplicate! Add 150 µL SOC to one and 250 µL to the other.
- Negative control: 2 µL of 5X diluted plasmid (5X dilution: 1 µL double digested p416tef1 + 4 µL sterile MQ water). This will transform 9,68 ng of linear empty plasmid.
- Plate both on LB + amp
- 37 degrees overnight

Replate and inoculate colony overnight

Replate 4 colonies (depending on amount on negative control) on 1 LB + amp plate. Dip green loop in liquid LB + amp media after you replated the colony. Number plates and tubes. Inoculate liquid tube overnight 37 degrees.

Miniprep
Check which plates has colonies and take corresponding tubes for miniprep. Follow protocol. Spin longer with the column empty to remove ethanol. Elute with 50 µL elution buffer.

Restriction verify
See last page

pPCK1

Prepare Gibson reaction

p416+pPCK1+GFP

	p416tef1 cut	pPCK1	GFP	Mastermix	Sterile MQ water
Conc fragment (ng/µL)	24,2	78,8	35	---	---
Volume (µL)	4	1	2	10	3
Volume half Gibson (µL)*	2	0.5	1	5	1.5

*"Half" Gibson reaction: The volumes of all components in the reaction are half of the regular Gibson reaction which is 20 µL in total. Saves Gibson master mix which is very expensive.

Gibson reaction
Run half the reaction, 20 µL of Gibson reaction is more than we need.
2 hours 50 degrees

Transform to *E. coli* (3 tubes of E. coli)
Refer to e. coli heat shock transformation protocol.
Use SOC instead of LB for the recovery stage after heat chock.

- Transforms 2 µL of Gibson assembly to *E. coli.* Do one with 2 µL and one with 5 µL.
- Negative control: 5 µL of 5X diluted plasmid (5X dilution: 1 µL double digested p416tef1 + 4 µL sterile MQ water). This will transform 24.2 ng of linear empty plasmid.
- Plate both on LB + amp
- 37 degrees overnight

Replate and inoculate colony overnight

Replate 4 colonies (depending on amount on negative control) on 1 LB + amp plate. Dip green loop in liquid LB + amp media after you replated the colony. Number plates and tubes. Inoculate liquid tubes and plates overnight 37 degrees.

Miniprep
Check which plates has colonies and take corresponding tubes for miniprep. Follow protocol. Spin longer with the column empty to remove ethanol. Elute with 50 µL elution buffer.

Restriction verify
See last page

pPYK2

Prepare Gibson reaction

p416+pPYK2+GFP

	p416tef1 cut	pPYK2	GFP	Mastermix	Sterile MQ water
Conc fragment (ng/µL)	24,2	12,5	35	---	---
Volume (µL)	4	4	2	10	0
Volume half Gibson reaction (microL)	2	2	1	5	0

*"Half" Gibson reaction: The volumes of all components in the reaction are half of the regular Gibson reaction which is 20 µL in total. Saves Gibson master mix which is very expensive.

Gibson reaction
Run half the Gibson reaction. 20 µL is more than we need.
2 hours 50 degrees

Transform to *E. coli* (3 tubes of E. coli)
Refer to e. coli heat shock transformation protocol.
Use SOC instead of LB for the recovery stage after heat chock.

- Transforms 2 µL of Gibson assembly to *E. coli.* Do duplicates, one with 2 µL and one with 5 µL.
- Negative control: 5 µL of 5X diluted plasmid (5X dilution: 1 µL double digested p416tef1 + 4 µL sterile MQ water). This will transform 24.2 ng of linear empty plasmid.
- Plate both on LB + amp
- 37 degrees' overnight

Replate and inoculate colony overnight

Replate 4 colonies (depending on amount on negative control) on 1 LB + amp plate. Dip green loop in liquid LB + amp media after you replated the colony. Number plates and tubes. Inoculate liquid tube overnight 37 degrees.

Miniprep
Check which plates has colonies and take corresponding tubes for miniprep. Follow protocol. Spin longer with the column empty to remove ethanol. Elute with 50 µL elution buffer.

Restriction verify
See last page

Restriction verify

Restriction reaction	
Sterile MQ water	Up to 10 µL
10X FastDigest **Green** Buffer	1 µL
Miniprepped construct	150 ng
SpeI (BcuI)	0.5 µL
SacI	0.5 µL
Total	10 µL

Incubate for 60 minutes 37 degrees.

Load on a prestained gel red gel. Run at 85V for approximately 1 hour.
Expected sizes:

1: assembled pAQR1 (5.2) SacI + SpeI 1. 5107 bp 2. **1415** bp	2: Assembled pFBP1 (5.3) SacI + SpeI 1. 5107 bp 2. **1692** bp	3: assembled pGLN1 (5.3) SacI + SpeI 1. 5107 bp 2. **1798** bp	4: Assembled pPCK1 (5.5) SacI + SpeI 1. 5107 bp 2. **1520** bp	5: Assembled pPYK2 (5.6) SacI + SpeI 1. 5107 bp 2. **1185** bp	6: p416tef (empty plasmid) SacI + SpeI 1. 5107 bp 2. **421** bp

Figure below: Visualization of the expected sizes.